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Determination of Sample Concentrations by PULCON NMR Spectroscopy

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Abstract

PULCON (Pulse Length Based Concentration Determination) is a powerful, versatile, non-invasive, and accurate technique for measuring solution concentrations during routine NMR spectroscopy. As solutes are quantified directly by their unique resonances, this technique avoids weight-based errors caused by contaminants (e.g. moisture), allows NMR samples to be directly employed in biological assays, and is particularly useful for quantifying small molecules, peptides, unstable molecules, and other materials that are difficult to weigh or handle. This article provides an introductory guide for biological and medicinal chemists, and highlights the diversity of applications.

Background

PULCON[1] (Pulse Length Based Concentration Determination) is a powerful, non-invasive, and accurate (> 98%) technique for measuring solute concentrations by quantitative NMR spectroscopy[2, 3] that is applicable to any NMR sensitive solute that can produce good quality NMR spectra. This article provides an introductory guide for biological and medicinal chemists, and highlights the diversity of applications.

PULCON can be conducted with standard NMR spectrometers without additional specialised equipment or software, and has many advantages, especially for preparing compound solutions for biological assays. Firstly, amounts of solute or solvent do not need to be measured, with individual solute concentrations directly quantified by their unique signals (resonances). This avoids weight-based errors caused by contaminants (e.g. moisture, salts) and counter-ions, and facilitates the solution preparation of viscous products or small amounts of material, which can be difficult to weigh. Secondly, internal standards are not needed, with concentrations determined by routine ^{1}H NMR spectroscopy (typically in DMSO-d_6 or 10 % D_2O/H_2O with water suppression). These allow the NMR sample to be directly employed in biological assays after spectral characterisation, particularly advantageous if the sample is scarce, unstable, or non-isolatable.

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Conflicts of Interest

JYWM is a named inventor on a patent describing 5-OP-RU and 5-OP-RU analogues as immunological reagents.

PULCON for most of the molecules encountered in biological and medicinal chemistry can be conducted as follows (Fig. 1). The ^{1}H NMR spectra of the sample of interest and a reference sample of known concentration (e.g. 15 mM benzoic acid in DMSO-d_6,[4] 10 mM sucrose in 10 % D_2O/H_2O,[1] or 40 mM cholesterol in $CDCl_3$),[5] should be measured using the same spectrometer (probe) and pulse program. The solutions should fully fill the active coil volume. Viscous samples should be avoided due to line broadening and reduced signal to noise. After tuning, matching, and shimming, both spectra should be recorded using the accurately determined 90° pulse lengths unique to each sample (see Fig. 2), the same receiver gain, and a long relaxation time of 30 seconds (sufficient to fully relax most ^{1}H nuclei[1, 6]). The temperature of the two samples can be different.

After careful phasing, baseline corrections, and checking for spectral quality (e.g. adequate signal to noise and solvent suppression [if required], no baseline distortions, see Fig. 3), a well-defined resonance should be integrated in each spectrum. Resonances that are broad (suggesting chemical or conformational exchange) or near solvent suppression should be avoided, as their integrations may be highly inaccurate (Fig. 3).

The solute concentration is then calculated using the adapted[1] equation below,[7] which relates concentrations (c) of the reference (R) and sample of interest (X) with the integration of the well-defined resonance (I), the number of protons corresponding to that resonance (A), the sample temperature in Kelvin (T), the 90° pulse length (θ), and the number of scans (n) (see Fig. 4), with f equal to 1 if receiver gain and the pulse program are the same for both samples.[8]

$$c_X = f c_R \frac{I_X A_R T_X \theta_X n_R}{I_R A_X T_R \theta_R n_X}$$

Importantly, the values of the reference sample can then be used for subsequent samples on the same probe and pulse program,[1] even if the reference was in a different solvent[9]. The 2D NMR extension[10] enables quantification of molecules with significantly overlapped resonances.

Applications

Recent applications are wide-ranging. For small molecules in biological and medicinal chemistry (Fig. 5), PULCON was used to prepare solutions of physicochemical probes (**1**[11] and **2**[12]) and radical scavengers (**3**)[13] for studying ligand-protein binding and oxidative stress, respectively. It was also used to analyse the DMSO solubility of 939 drug fragments,[14] and to support entire drug discovery campaigns (e.g. **4, 5** and **6**),[15–17] demonstrating its capacity to facilitate rapid generation of structure-activity relationships.

Metabolite mixtures from bacterial (**7** – **11**)[18] and cancer cells (**7, 12** – **14**)[19] could be spectroscopically quantified (Fig. 6). Coffee quality and authenticity were monitored via PULCON after $CDCl_3$ extraction of natural products from coffee grounds (**15** – **19**, Fig. 7).[20]

Meanwhile, PULCON measurements of the structurally complex and scarce toxin okadaic acid (**20**) through multiple separate resonances (via ERETIC2)[7] were each consistent with an internal standard method (Fig. 8).[9]

For peptides, charged sidechains are often accompanied by unknown numbers of counter-ions (e.g. trifluoroacetates from HPLC eluants), which obfuscates their true molecular weights. PULCON bypasses this problem, and is typically performed in DMSO or 10 % D_2O/H_2O through integration of known numbers of amide protons, which are far away from the suppressed water resonance.[21] This has enabled the systematic quantification of diverse peptides, including long constrained peptides (e.g. **21**),[22] cyclic lipopeptides (e.g. **22**),[23] and cyclic pentapeptides (e.g. **23**)[24] for biological assays and structural calculations via circular dichroism spectroscopy (Fig. 9). Hydroxyl groups in structurally complex lignin biopolymers (**24**) were quantified by *in situ* hydroxyl phosphitylation in pyridine/$CDCl_3$ followed by PULCON on the ^{31}P nuclei,[25] illustrating generality across NMR sensitive nuclei (Fig. 9).

PULCON is especially useful for unstable molecules. After suspending a mixture of oxidation prone amine 5-A-RU (**25**) and inorganic stabilisers in DMSO-d_6, followed by filtration of stabilisers, PULCON of **25** enabled the precise addition of methylglyoxal (1.1 eq) to yield bacterial metabolite 5-OP-RU (**26**, Scheme 1).[27] This compound is highly prone to hydrolysis and cyclisation in water (to **27**) and cannot be readily purified,[27] but PULCON critically enabled the NMR sample to be used as a potent immunostimulant[28] for protecting mice against bacterial infections[29–31] and tumours[32].

Similarly, its water stable analogue JYM72 (**28**) is unstable when neat (Scheme 1).[27] However, partial concentration of its aqueous HPLC eluants,[27] accurate dilution into 10 % D_2O/H_2O, and then PULCON, enabled its preparation as a cancer immunotherapy tool[32]. Together, these exemplify PULCON as a powerful solution quantification technique for biological and medicinal chemistry.

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Biography

Jeffrey Mak was awarded a University Medal (University of Queensland) before undertaking his PhD in total synthesis with Prof. Craig Williams. He joined the Fairlie group at the Institute for Molecular Bioscience, where his interests include biological chemistry, chemical biology, and medicinal chemistry. In 2014, he was part of a multidisciplinary Australian team that discovered the unstable microbial metabolites (e.g. 5-OP-RU) that potently activate mucosal associated invariant T cells. Dr Mak was selected as a CAS

SciFinder Future Leader (2017), lectures undergraduate synthetic chemistry at UQ, and was recently promoted to Research Fellow.

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[6]. While a delay of 30 seconds is more than sufficient for the majority of samples, the delay should be increased if integrations do not scale with number of scans. For information on the importance of relaxation time constants in quantitative NMR, how they can be measured, and example values for various nuclei, seeWei R, Dickson CL, Uhrín D, Lloyd-Jones GC, Journal of Organic Chemistry 2021, 86, 9023.

[7]. Users could also use ERETIC2, which is a potentially convenient PULCON tool built into Bruker TopSpin NMR processing software. Refer to: Tyburn J-M, Coutant J. Electronic to Access In-Vivo Concentration User Manual 2016 (Bruker).

[8]. The factor f accounts for signal intensity differences between the reference and the sample of interest if the two spectra were obtained using different pulse programs and receiver gains.

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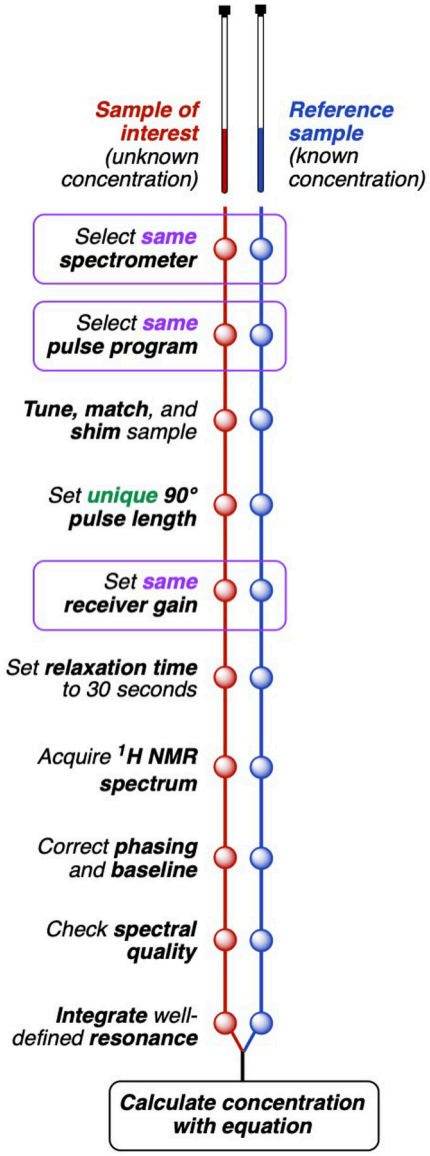


Figure 1.
Flowchart of NMR sample concentration determination using PULCON.

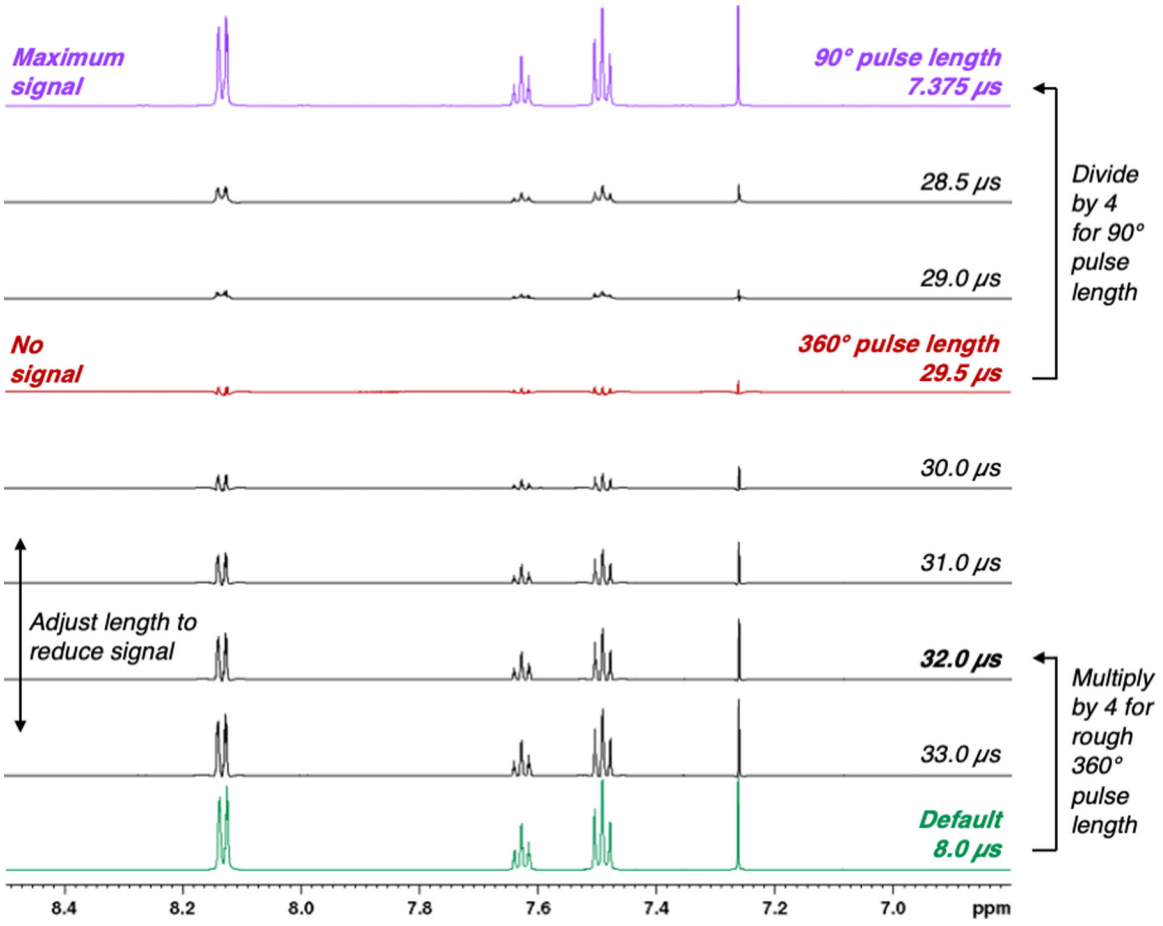


Figure 2.
The 90° pulse length can be obtained by dividing the accurately measured 360° pulse length (no signal) by 4.

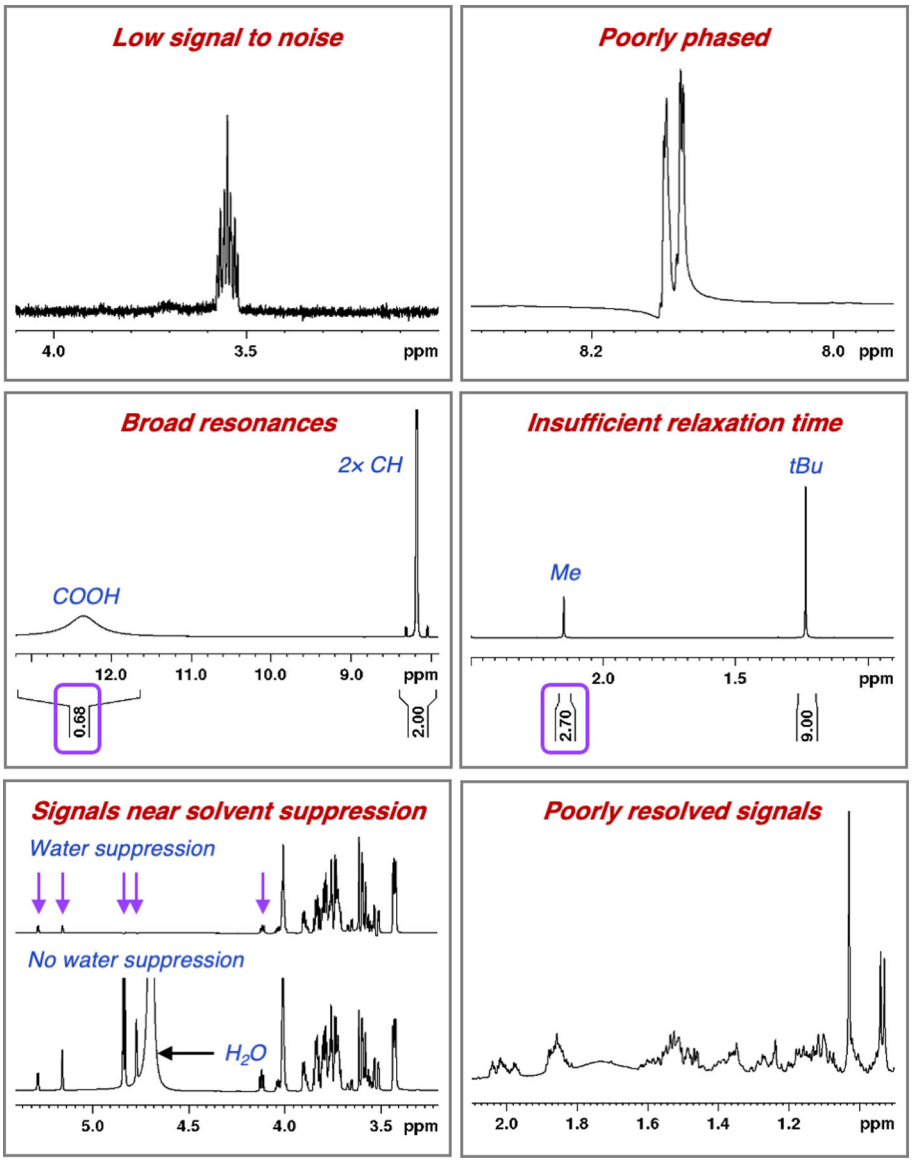


Figure 3.
Instances where PULCON may be inaccurate.

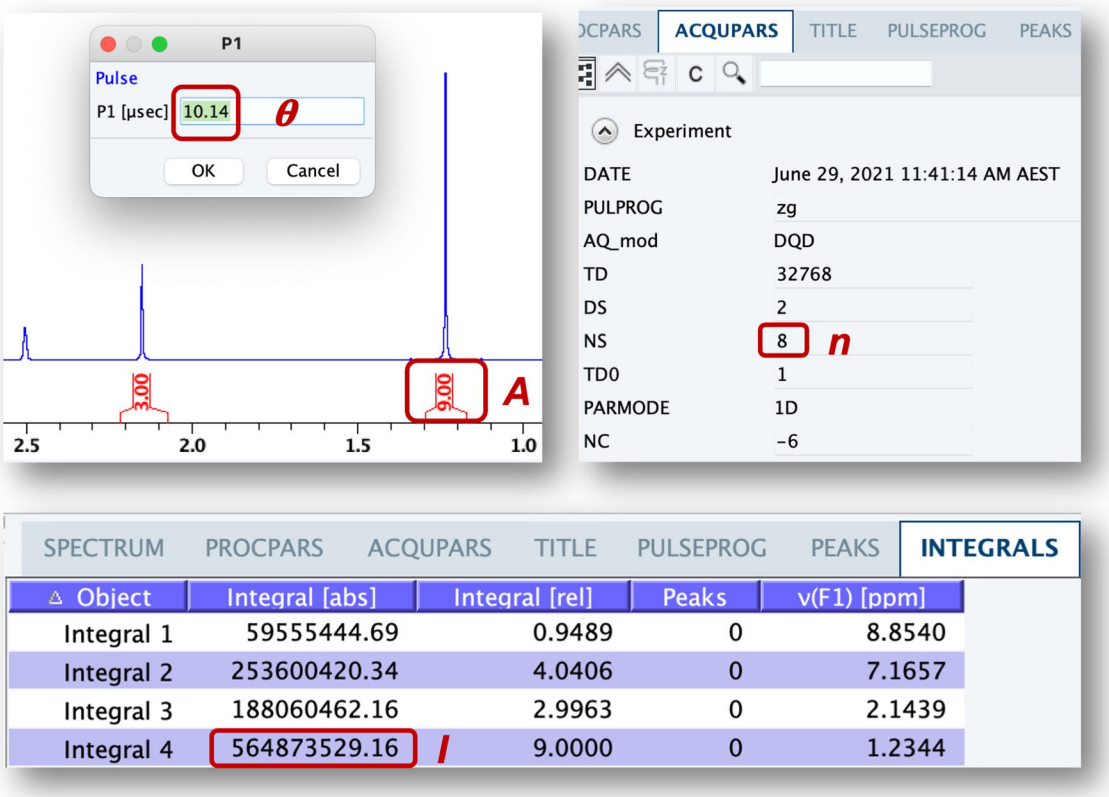


Figure 4.
PULCON parameters and values are readily obtained from NMR software after spectroscopic analysis (TopSpin 4.02 shown).

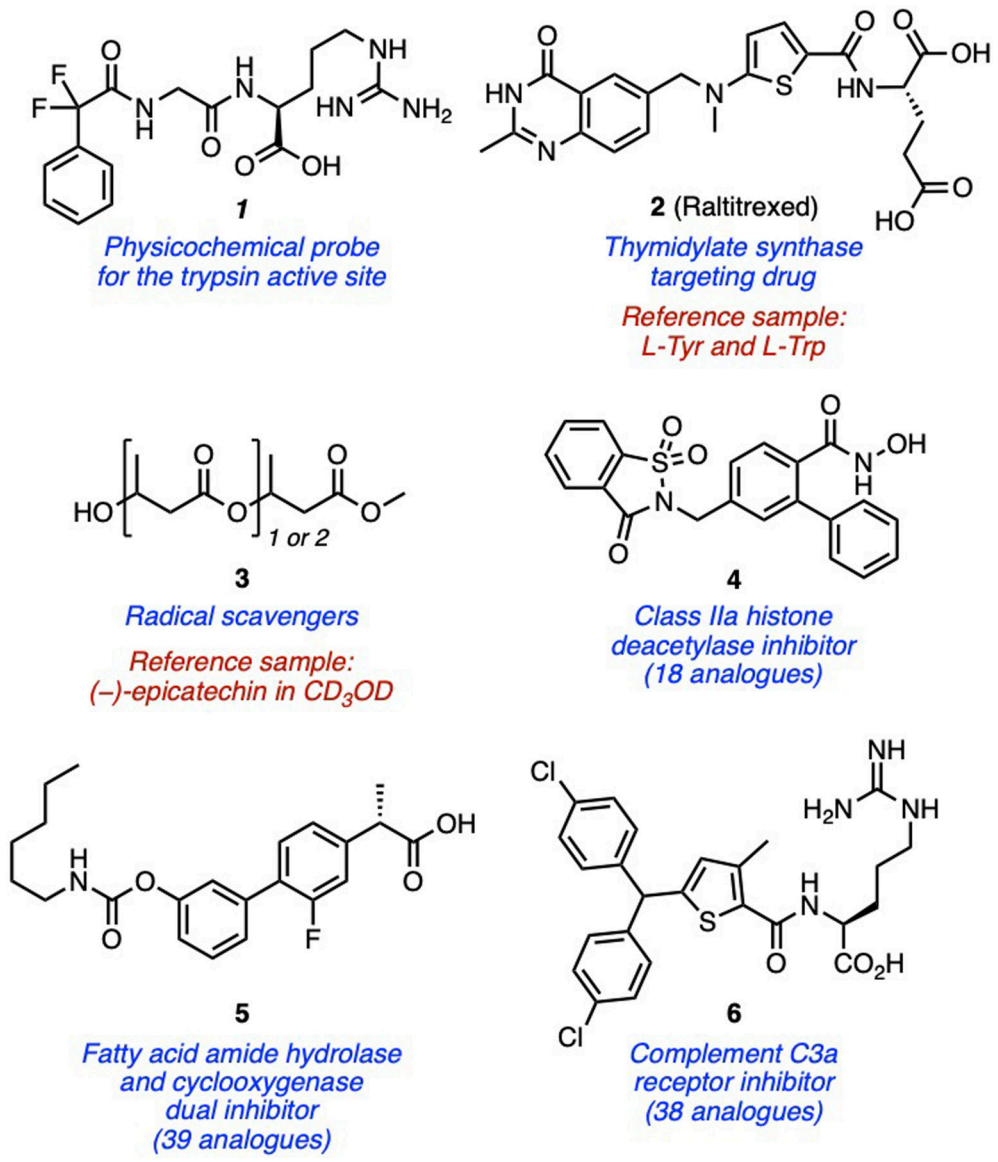


Figure 5.

Recent examples of PULCON quantification of small molecules in biological and medicinal chemistry.

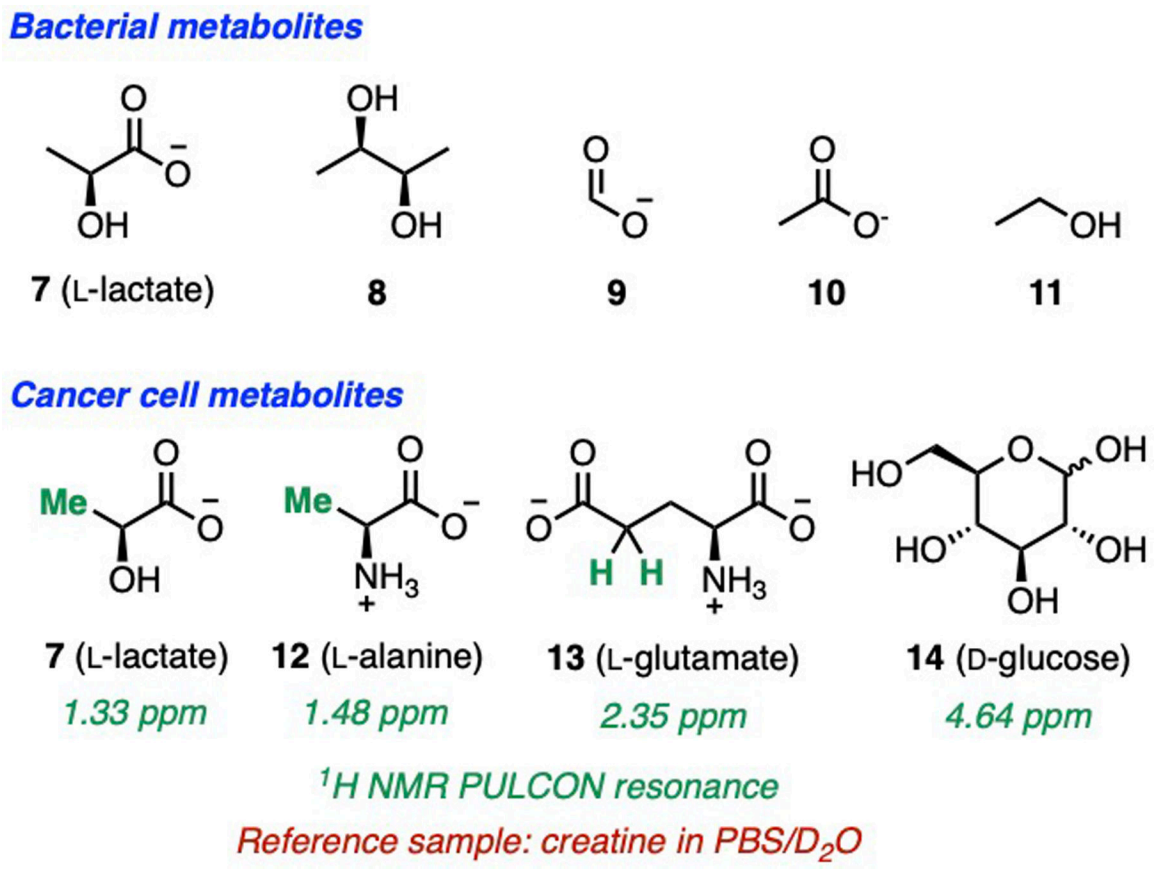


Figure 6.
PULCON quantification of biological mixtures.

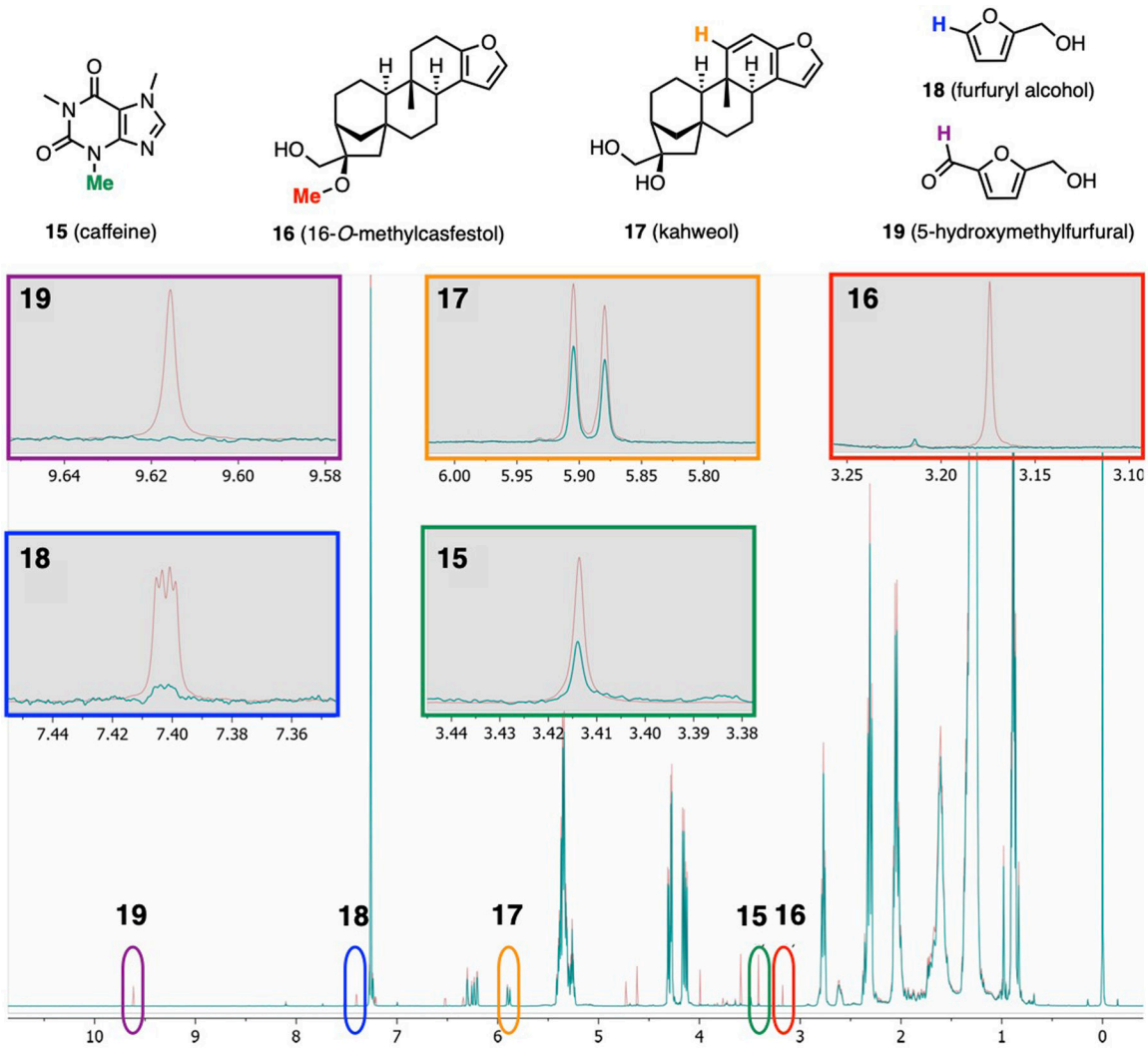


Figure 7.

PULCON quantification of a coffee natural product mixture (teal trace) through marked resonances selected from spectra of pure **14** – **18** (maroon trace). Annotated ^{1}H NMR spectrum adapted from reference [20].

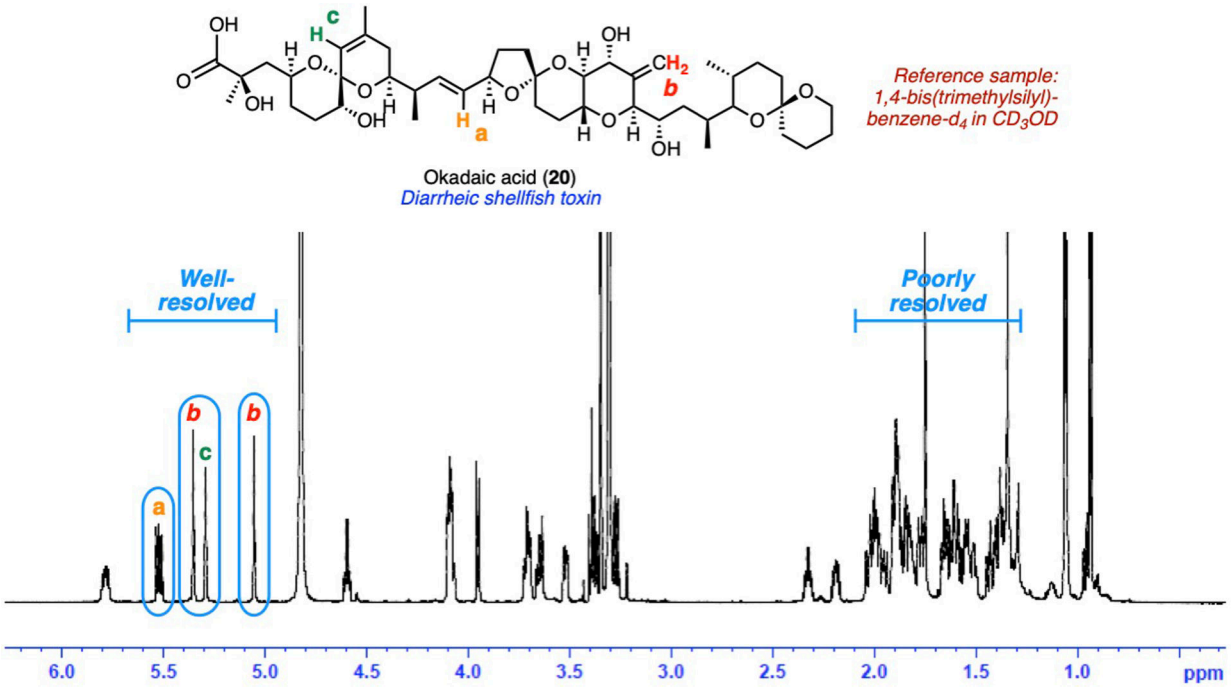


Figure 8.

PULCON quantification of scarce toxin okadaic acid. Annotated ^{1}H NMR spectrum adapted from reference [9].

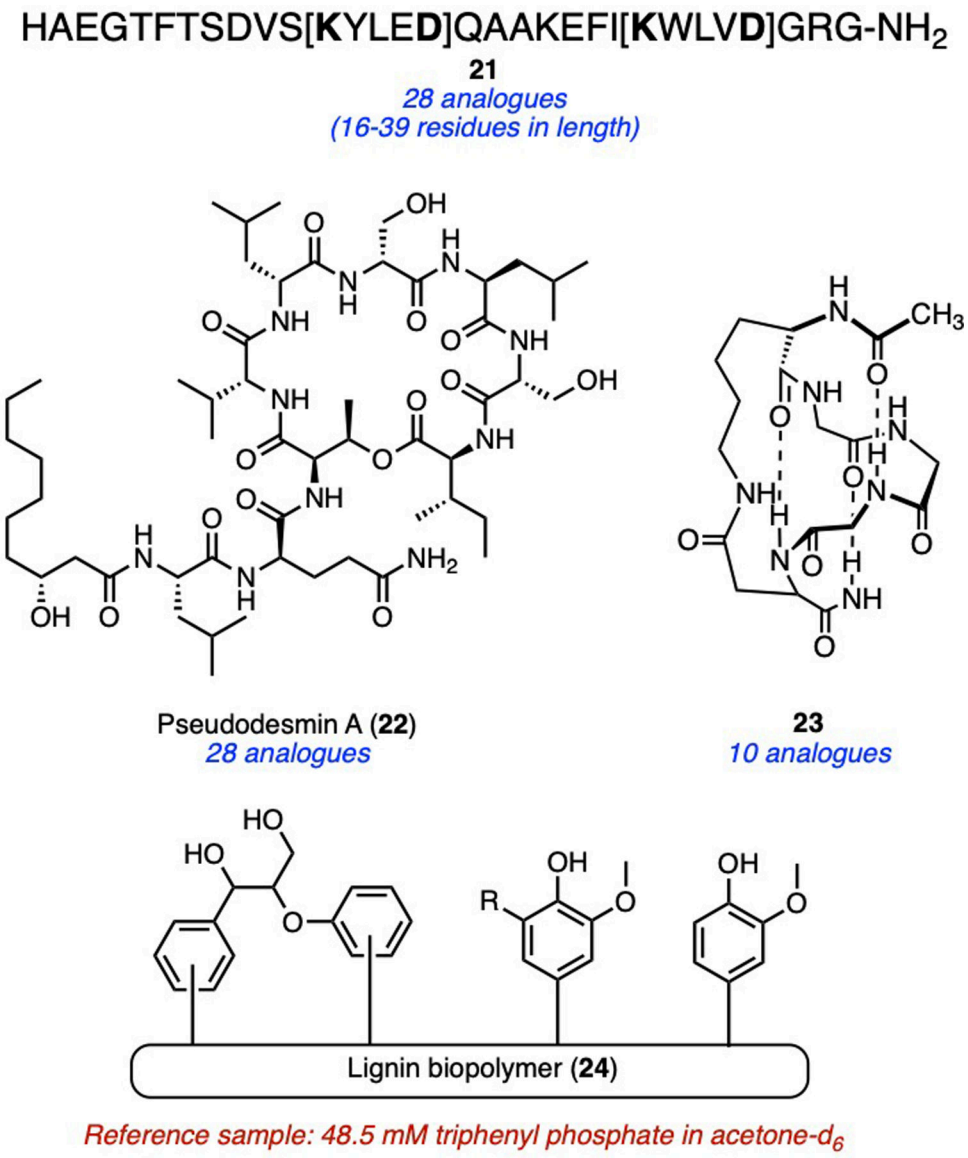
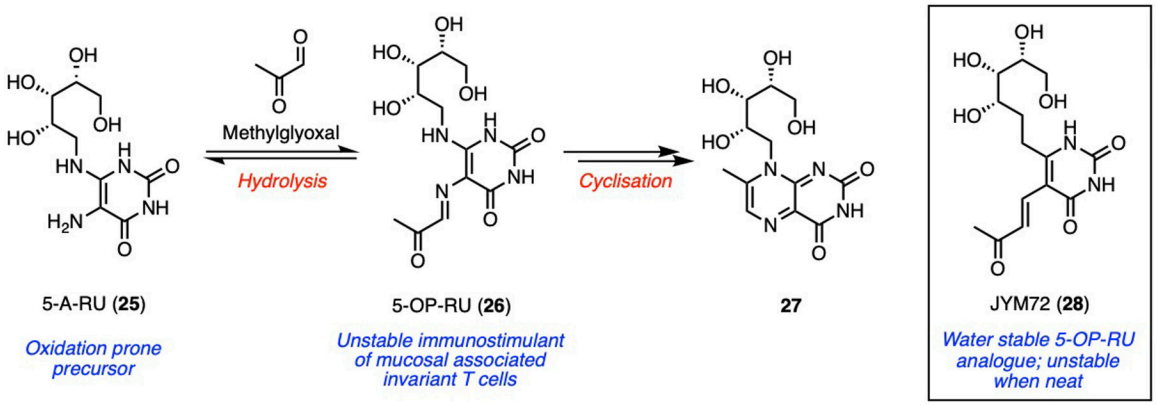


Figure 9.
Recent examples of diverse peptides and other biopolymers quantified by PULCON.



Scheme 1.

PULCON enabled *in situ* quantification of unstable precursor 5-A-RU (**25**), and unstable immunostimulants 5-OP-RU (**26**) and JYM72 (**28**).